many laboratories will have most of their constructs in GFP form and entire genomes are available as functional GFP-fusion proteins.
Here, we report a method that makes all these constructs available for superresolution microscopy by targeting GFP with tiny, high-affinity antibodies coupled to blinking dyes. It thus combines the molecular specificity of genetic tagging with the high photon yield of organic dyes and minimal linkage error, as demonstrated on microtubules, living neurons and yeast cells. We show that in combination with GFP-libraries, virtually any known protein can immediately be used in superresolution microscopy and that high-throughput superresolution imaging using simplified labeling schemes is possible.
The labeling density in superresolution microscopy based on photoactivatable fluorophores is limited by the fact that a small, but significant fraction is always in the bright state. To overcome this limitation we implemented binding-activated localization microscopy (BALM), which is based on the localization of individual binding events of fluorophores that show a fluorescence enhancement upon binding to their target structures. Using nucleic acid stains on double-stranded DNA we yielded a resolution of ~14 nm (fwhm) and a spatial sampling of 1/nm in vitro and could visualize the organization of the bacterial chromosome in fixed Escherichia coli cells. In general, the principle of binding-activated localization microscopy can be extended to other dyes and targets such as protein structures.

51-Subg
Simultaneous Imaging of Vesicle Trafficking and Calcium-Mediated Exocytosis in Pancreatic Beta-Cells
David W. Piston[1], Amicia D. Elliott[2], Alessandro Ustione[2].
[1]Biophysics, Vanderbilt Univ, Nashville, TN, USA, [2]Vanderbilt Univ, Nashville, TN, USA.
It has long been known that only a small fraction (<10%) of the insulin in pancreatic beta-cells can be released. Clinical treatments of type 2 diabetes focus on increasing insulin release, so an understanding of insulin vesicle trafficking and release may lead to novel therapeutic strategies. Towards this understanding, we have utilized a novel quantitative imaging assay, based on a double fusion to the integral vesicle protein phogrin. In this construct, a pH-dependent ecliptic pHluorinFP is inside the lumen of the granule and a mOrangeFP is outside the granule in the cytoplasm. The pHluorin fluorescence is quenched by the low pH inside the granule until exocytosis when the lumen of the granule mixes with the extracellular media. This increases the local pH and the green fluorescence can then be measured as a readout of exocytosis. Experiments were done on the multicolor TIRF system with a photoactivation module. We photoconverted the mOrange-phogrin to a deep red protein in the perinuclear region, and then watched where that population of granules went as we stimulated the cells with glucose. Having only a small subset of vesicles labeled greatly facilitates tracking. We used this approach with TIRF microscopy to measure secreted granules and determine if they came from previously docked granules or from regions beyond the TIRF imaging field. In beta-cells, we find that secreted vesicles do not come from a docked pool, with secretion happening within about 1 second of the vesicle's arrival at the membrane. This time is decreased even further with the addition of glucose. These data suggest that the releasable insulin pool in beta-cells may not be docked at the membrane as are synaptic vesicles, but rather are maintained deeper with in the cell.

52-Subg
Invasive Optics for Watching the Brain Work
Michael Levene.
Yale University, New Haven, CT, USA.
Optical methods are revolutionizing neuroscience, from high-resolution multiphoton microscopy, to functional imaging with Ca^{2+}- and voltage-sensitive dyes, to direct optical stimulation and inhibition of neurons with light. Yet this incredibly powerful toolbox has been limited in its application by an inability of microscopy to image deep in the living brain. We have made great strides in this direction through novel invasive micro-optics capable of penetrating the brain to gain access to deep brain regions. We will show recent results, including simultaneous Ca^{2+}-sensitive imaging of hundreds of neurons throughout all 6 layers of cortex in awake mice.

53-Subg
Assembly and Dynamics of Nucleic Acid - Protein Complexes at the Single-Molecule Level
David Millar.
Scripps Research Inst, La Jolla, CA, USA.
Single-molecule fluorescence spectroscopy is emerging as a powerful tool for detailed biophysical analyses of nucleic acid - protein interactions, because of the ability to resolve different binding modes and to provide kinetic information on protein conformational changes during assembly or biological function. To illustrate these capabilities, I will describe two systems currently under study in my laboratory. (1) The HIV-1 protein Rev mediates the nuclear export of unspliced and partially spliced mRNAs encoding viral structural proteins. Rev interacts with a highly conserved element within the viral pre-mRNA known as the Rev response element (RRE). Multiple Rev monomers must assemble on the RRE, mediated by a combination of RNA-protein and protein-protein interactions. Multi-color single-molecule TIRF microscopy is used to monitor hundreds of individual Rev-RRE assembly reactions in parallel, revealing the mechanism of oligomeric assembly and the influence of cellular cofactors on the assembly pathway. (2) DNA polymerases replicate DNA substrates with extraordinarily high fidelity because of their ability to discriminate between cognate and non-cognate nucleotide substrates during each cycle of nucleotide incorporation and to remove misincorporated bases using a separate proofreading activity. Single-molecule FRET methods are used to observe conformational changes of the fingers subdomain of a model DNA polymerase during the process of selection of incoming nucleotide substrates, revealing a novel ''ajar'' conformation that acts as a fidelity checkpoint before the fingers enclose the nascent base pair. These methods are also used to monitor the movement of the nascent DNA strand during proofreading, revealing that the DNA can switch between the spatially separated polymerase and exonuclease sites while remaining bound to the enzyme. These observations provide new insights into the important role of enzyme conformational dynamics during the processes of nucleotide substrate selection and proofreading.

Subgroup: Membrane Structure & Assembly

54-Subg
Discovering Highly Potent Pore-Forming Peptides using Synthetic Molecular Evolution
Aram J. Krauson, Jing He, **William Wimley**.
Tulane, New Orleans, LA, USA.
There are many natural and designed peptides that permeabilize membranes, and there are multiple mechanisms by which membrane permeabilization can occur. Yet, peptides that unequivocally self-assemble into equilibrium, membrane-spanning pores at low peptide to lipid ratios (P:L1:1000) are very rare. The design and engineering of such peptide ''nanopores'' in lipid bilayer membranes is desirable as it could lead to improved biosensor platforms, targeted therapeutics, exogenous ion channels, or drug delivery vehicles. While the few well studied pore-forming peptides have provided a lot of information about the architecture of peptide pores, especially -helical pores, our knowledge of the fundamental molecular principles of pore formation is not detailed enough for rational engineering. This is a roadblock to the design of new pore-forming peptides and to the optimization of known pore-formers for particular applications. In this work we show how novel, highly potent, equilibrium pore-forming peptides can be discovered using synthetic molecular evolution, i.e. iterative cycles of combinatorial library design and high-throughput screening. In the first example, we used two generations of *de novo* library design and screening to identify highly potent pore-formers that self-assemble into -sheets in membranes. These peptides may be the only known examples of highly potent, pore-forming peptides that have -sheet secondary structure in membranes. In the second example we designed an iterative library that used the helical pore-former melittin as a template. From this library we identified gain-of-function pore-formers that are much more potent than melittin. The results demonstrate the power of synthetic molecular evolution for the discovery and engineering of membrane active peptides.

55-Subg
Exploring the Mechanisms of Antimicrobial Lipopeptides with Molecular Simulation
Alan Grossfield, Ph.D.[1], Joshua N. Horn[2], Tod D. Romo[2], Dejun Lin[2], Aaron Cravens[2].
[1]Biochemistry & Biophysics, University of Rochester Med School, Rochester, NY, USA, [2]University of Rochester Med School, Rochester, NY, USA.
Antimicrobial peptides (AMPs) — small peptides that kill bacteria and fungi by attacking their outer membranes — have been touted as a possible solution to the emergence of new strains of antibiotic-resistant pathogens. Although some AMPs have become promising drug candidates, overall they have not been especially successful clinically, primarily because of their size and bioavailability. To avoid these difficulties, we have focused our efforts on smaller peptides chemically modified to include acyl chains at their N-termini; the fatty acid chains give the peptides the ability to bind membranes efficiently without including a large number of hydrophobic side chains. Using a combination of

all-atom and coarse-grained molecular dynamics simulations, we have explored the membrane-binding behavior of several lipopeptide families, including the Shai peptides, Lfb6, and fengycins. These simulations, validated via careful comparison with experiment, help to reveal the atomic-level mechanism for their function, yielding insights needed for rational design of better antibacterial and antifungal agents.

56-Subg
Fatty Acids and Lysolipids Perturb Lipid Membranes: Implications for Drug Delivery
Ole Mouritsen[1], Ahmad Arouri[2].
[1]Memphys Ctr Biomemb Phys, MEMPHYS, University Southern Denmark, Odense, Denmark, [2]MEMPHYS, University Southern Denmark, Odense, Denmark.
Fatty acids and lysolipids incorporate into lipid membranes and may exert an effect on their permeability, morphology, and stability, leading, e.g., to a reduction in the permeability barrier. The origin of this phenomenon may be related to changes in the curvature stress of the membrane caused by the effective non-cylindrical geometry of fatty acids and lysolipids as compared to cylindrical phospholipids. It has been proposed that the same effects may carry over to apply for the permeability barrier of cell membranes, in which case the effect could possibly be exploited to enhance intracellular drug uptake. However, fatty acids and lysolipids are in themselves cytotoxic in micromolar concentrations and can induce cell lysis and apoptosis. Experiments with living cells have shown that fatty acids and lysolipids at concentrations below their cytotoxicity limit cannot render cell membranes more permeable by perturbing the lipid-bilayer component of the membrane. This implies that development of liposomal drug-delivery systems, e.g., those using endogenous phospholipase activity as a trigger to unload drugs, are faced with the problem of overcoming the barrier for transferring active drugs across the target membranes.
References:
T. L. Andresen, S. S. Jensen, and K. Jørgensen, Prog. Lipid Res. 44, 197-224 (2005); N. Rasmussen, N., J. H. Andersen, H. Jespersen, O. G. Mouritsen, and H. J. Ditzel, Anticancer Drugs 21, 674-677 (2010); A. Arouri and O. G. Mouritsen, J. Liposome Res. 21, 296-305 (2011); A. Arouri and O. G. Mouritsen, Eur. J. Pharm. Sci. 45, 408-420 (2012); H. Jespersen, J. H. Andersen, H. J. Ditzel, and O. G. Mouritsen, Biochimie 94, 2-10 (2012); A. Arouri and O. G. Mouritsen, Prog. Lipid Res. in press (2012).

57-Subg
Both Detergent Effects Upon Domain Size and Transmembrane Protein Length Effects Upon Domain Binding Suggest that Hydrophobic Mismatch can Control the Properties of Ordered Membrane Domains ("Rafts")
Erwin London.
Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NM, USA.
Hydrophobic mismatch between co-existing domains potentially plays an important role in determining domain size and domain interaction with transmembrane proteins. We recently found that in bilayers with the potential to form co-existing Ld and Lo domains, the detergent Triton X-100 enlarges ordered domain size without perturbing ordered domain formation. This can be explained if the effect of detergent is greatest at the boundary between Lo and Ld domains, which would be the case if the main effect of detergent bound to the membranes is to alter the difference between Lo and Ld bilayer width. In other studies, we found that for a multi-transmembrane segment protein, perfringolysin O (PFO), altering the length of transmembrane sequences controls affinity of the protein for membranes domains in a fashion that is dependent upon domain bilayer width. In bilayers with co-existing Ld and Lo domains, PFO with shortened transmembrane segments preferred to partition into the membrane domains with a thin bilayer width, while PFO with lengthened transmembrane segments preferred to partition into domains forming a wider bilayer. PFO with intermediate length, wild type, transmembrane segments exhibited intermediate behavior. This relative bilayer width preference was the same whether the Lo domains were the thinner or wider membrane domains. The effect of transmembrane length upon domain localization was observed both in vesicles that have domains large enough to see by light microscopy and in vesicles with sub-microscopic domains, in which domain affinity was assayed with FRET. Thus, for both of detergent effects and protein-domain association experiments it is likely that hydrophobic mismatch is a key parameter.

58-Subg
Functional Reconstitution of Membrane Proteins by Isothermal Titration Calorimetry
Sandro Keller, Jun. Prof. Dr.[1], Nadin Jahnke[2], Oxana Krylova[2], Carolyn Vargas[3].
[1]Molecular Biophysics, University of Kaiserslautern, Kaiserslautern, Germany, [2]Leibniz Institute of Molecular Pharmacology (FMP), Berlin, Germany, [3]University of Kaiserslautern, Kaiserslautern, Germany.
Membrane proteins make up roughly 30% of all proteins encoded by the human genome and represent about 50% of drug targets in the human body. They fulfil vital functions as receptors and signal transducers, channels and transporters, motors and anchors. Many of these functions are amenable to biochemical and biophysical investigation only after the membrane protein of interest has been extracted, purified, and reconstituted into artificial liposomes. Extraction from the host-cell membrane and chromatographic purification are usually performed with the aid of detergents. However, detergent micelles do not allow the study of vectorial functions such as solute transport or signal transduction. Therefore, numerous membrane proteins need to be reconstituted from a purified, detergent-solubilised state into liposomes in order to regain their native structures and activities.
Unfortunately, functional reconstitution has remained one of the main bottlenecks in the handling of membrane proteins. In particular, gauging the success of reconstitution experiments has thus far been limited to trial-and-error approaches. To address this problem, we have established high-sensitivity isothermal titration calorimetry (ITC) as a powerful method for monitoring the reconstitution of membrane proteins into liposomes. ITC has previously been employed for characterising liposome solubilisation and reconstitution in the absence of protein [1,2]. Recent work in our laboratory has demonstrated that ITC is also excellently suited for tracking the complex process of membrane-protein reconstitution in a non-invasive and fully automated manner. The approach is exemplified for the prokaryotic potassium ion channel KcsA, which was functionally reconstituted into stable proteoliposomes at high protein densities. Electrophysiological experiments confirmed that KcsA regained its functional activity upon ITC-guided reconstitution.
[1] Heerklotz, Tsamaloukas, Keller. 2009. Nat. Protoc. 4: 686-697.
[2] Krylova, Jahnke, Keller. 2010. Biophys. Chem. 150: 105-111.

59-Subg
Detergents for Extraction, Purification, and Reconstitution of G Protein-Coupled Membrane Receptors
Klaus Gawrisch, Alexei A. Yeliseev, Tomohiro Kimura, Olivier Soubias.
LMBB, NIAAA, NIH, Bethesda, MD, USA.
G protein-coupled membrane receptors (GPCR) are, perhaps, the most important signaling molecules for the transfer of external signals to the cell interior. The rhodopsin-like receptors are the largest class of GPCR. They are integral membrane proteins with seven transmembrane helices, including not only receptors for vision, but also receptors for neurotransmitters, chemokines, neuropeptides, cannabinoids, lysolipids, prostaglandins, histamines, and odorants, just to mention a few. A still rather limited number of rhodopsin-like GPCR have been successfully overexpressed in various expression systems, solubilized with the help of detergents, purified in quantities up to milligrams, and reconstituted into lipid bilayers for functional and structural studies. Every one of those steps requires the use of detergents. Detergents are also used for crystallization of GPCR, although this will not be subject of my presentation. All GPCR are known to be highly vulnerable to denaturation while solubilized with detergents. The successful laboratories have spent years optimizing experimental conditions to increase yield of purified GPCR. A significant fraction of this process is identifying proper detergents for work with a particular GPCR. Most of this expertise was gained by trial and error. By now, patterns in the use of detergents have emerged that are applicable to more than one of the GPCR. In my talk, I will present experience gained with rhodopsin and the recombinant, type II cannabinoid receptor at my laboratory, but will also review literature on the use of detergents for other GPCR. I hope that my attempt of summarizing practical observations on GPCR and detergent use will stimulate discussions that eventually lead to a more purposeful selection of detergents in the future.

Subgroup: Exocytosis & Endocytosis

60-Subg
Molecular Basis and Physiological Consequences of Synaptic Vesicle Pool Heterogeneity
Ege Kavalali.
U.T. Southwestern Med. Center, Dallas, TX, USA.
In this presentation I will discuss our recent studies on the molecular basis and physiological consequences of synaptic vesicle pool heterogeneity.